


## STANDARD ARTICLE

# Prevalence of *Clostridium perfringens netE* and *netF* toxin genes in the feces of dogs with acute hemorrhagic diarrhea syndrome

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**Background:** Recently, novel pore-forming toxin genes designated *netE* and *netF* were identified in a *Clostridium perfringens* type A strain isolated from a dog with acute hemorrhagic diarrhea.

**Objectives:** Pore-forming toxins could play an important role in the disease pattern of acute hemorrhagic diarrhea syndrome (AHDS) in dogs. Thus, we aimed to determine the prevalence of *C. perfringens* genes encoding for *netE* and *netF* in the feces of dogs with AHDS and to evaluate any association between selected clinical variables and the presence of these toxin genes.

**Animals:** In total, 174 dogs were included in the study.

**Methods:** Fecal samples of all dogs were tested by real-time polymerase chain reaction for *netE* and *netF* genes. Time to recovery, hospitalization time, and selected laboratory variables were compared between dogs with AHDS that were positive or negative for the toxin genes.

**Results:** A significant difference was found among the 3 groups in the prevalence of the pore-forming toxin genes *netE* and *netF*: dogs with AHDS: 26 of 54 (48.1%); dogs with canine parvovirus (CPV) infection: 0 of 54 (0%); and healthy dogs: 8 of 66 (12.1%;  $P < .001$ ). In dogs with AHDS, no significant difference was detected in any variables evaluated between *netE*-positive and *netF*-positive and *netE*-negative and *netF*-negative dogs.

**Conclusions and Clinical Importance:** The prevalence of *C. perfringens* encoding for *netE* and *netF* is significantly higher in dogs with AHDS compared to control dogs. Further studies are warranted to evaluate whether these toxins are an inciting cause for AHDS in dogs.

## KEYWORDS

bacterial overgrowth, canine, hemorrhagic gastroenteritis, intestinal lesions, pore-forming toxin

## 1 | INTRODUCTION

Acute onset hemorrhagic diarrhea in dogs is a commonly observed syndrome with numerous potential causes.<sup>1</sup> By ruling out known causes of hemorrhagic diarrhea, a tentative diagnosis of “acute hemorrhagic diarrhea syndrome” (AHDS) can be made.<sup>2</sup> Although the specific etiology has not yet been fully elucidated, it has been suggested that *Clostridium perfringens* and their toxins are involved in the pathogenesis of AHDS.<sup>2</sup>

**Abbreviations:** AHDS, acute hemorrhagic diarrhea syndrome; *cpa*, *Clostridium perfringens* alpha toxin gene; *cpe*, *Clostridium perfringens* enterotoxin gene; CPV, canine parvovirus; qPCR, quantitative polymerase chain reaction; *tcp*, transfer of clostridial plasmids.

Quantitative PCR (qPCR) showed a significant increase of *C. perfringens* in the feces of dogs with AHDS compared to healthy dogs.<sup>3</sup> In intestinal biopsy specimens of dogs with AHDS, *C. perfringens* could be detected adherent to the surface of necrotic epithelial lesions.<sup>2</sup> This form of destructive enteritis associated with *C. perfringens* toxins also has been observed in other species.<sup>4–6</sup> Although it is very suggestive that *C. perfringens* is responsible for AHDS, it is still unclear which *C. perfringens* toxins cause the epithelial lesions.<sup>2,7</sup>

An unresolved question is whether *C. perfringens* overgrowth is a cause or a consequence of the underlying intestinal disease.<sup>2</sup> *C. perfringens* species may proliferate secondary to disruption of the normal gastrointestinal microbiota and might only be able to adhere

to the intestinal mucosa after destruction of the intestinal lining by other primary causes such as viral agents.<sup>8</sup>

*Clostridium perfringens* is an important gram-positive anaerobic bacterium that is found ubiquitously in the intestine of humans and animals.<sup>5</sup> Most of these bacteria are nonpathogenic in the intestine, but certain strains potentially can cause disease in humans and animals (eg, enteritis, enterotoxemia, and food poisoning).<sup>5,9</sup> Causative factors for this pathogenicity of *C. perfringens* are specific extracellular toxins,<sup>6,10,11</sup> all of which, except for the alpha toxin, are encoded on *tcp* (transfer of clostridial plasmids)-conjugative virulence plasmids.<sup>12</sup> Recently, *C. perfringens* type A strains encoding novel pore-forming toxins, designated *netE*, *netF*, and *netG*, were isolated from a dog with necrotizing gastroenteritis.<sup>4</sup> *NetE* and *netF* genes were present on a large *tcp*-conjugative plasmid, and *netG* was located on another large *tcp*-conjugative plasmid.<sup>13</sup> A significant association was found between *netF*-encoding *C. perfringens* and fatal cases of both hemorrhagic enteritis in dogs and necrotizing enteritis in foals.<sup>4</sup>

Therefore, our aim was to evaluate an association between the *netE* and *netF* pore-forming toxin genes and AHDS in dogs. For this purpose, the prevalence of *C. perfringens* type A encoding these pore-forming toxin genes was compared among dogs with AHDS, healthy dogs, and dogs with canine parvovirus (CPV) infection by qPCR of genomic DNA isolated from fecal samples. Furthermore, time of hospitalization, time to recovery, and selected laboratory variables were compared between *netE*-positive and *netF*-positive and *netE*-negative and *netF*-negative dogs within the AHDS group. Dogs with CPV infection were included as a 2nd control group to determine whether hemorrhagic diarrhea independent of the underlying disorder is associated with overgrowth of these toxinogenic *C. perfringens* strains.

## 2 | MATERIALS AND METHODS

### 2.1 | Study design

Our study used data and materials collected prospectively for other studies already completed and published, at a time before the *NetE*, *NetF*, and *NetG* pore-forming toxins were discovered.<sup>2,14–16</sup> All dogs were presented to Clinic of Small Animal Internal Medicine, Ludwig-Maximilian-University, Munich, Germany, between 2006 and 2014.

Prospective collection and analysis of fecal samples from dogs was approved by the Ethics Committee of the Centre of Veterinary Medicine, Ludwig-Maximilian-University, Munich, Germany (approval number 7-20-06-13).

### 2.2 | Study population

Inclusion and exclusion criteria for dogs in the study population were previously described.<sup>16</sup> A brief summary is given below.

#### 2.2.1 | Dogs with AHDS

The inclusion criterion was the presence of acute hemorrhagic diarrhea. Dogs diagnosed with any disease known to cause hemorrhagic diarrhea were excluded from the study. The following tests were conducted to detect underlying disorders and potential complications for

hemorrhagic diarrhea: CBC, serum biochemistry profile, fecal examinations for nematode parasites, and *Giardia* spp. CPV infection was ruled out by a negative fecal PCR test result in suspicious cases. In addition, serum bile acid concentrations, clotting profile, and medical imaging were performed at the discretion of the clinician, depending on clinical presentation and course of disease.

#### 2.2.2 | Healthy controls

Clinically healthy, client-owned dogs that were presented for routine vaccination to the healthcare service or that belonged to students or employees of the Clinic of Small Animal Medicine of the Ludwig-Maximilian-University Munich were included in the healthy control group. Dogs were excluded if they had any history of gastrointestinal disorders, such as vomiting, diarrhea, or anorexia up to 4 weeks before to sampling. In addition, detection of nematode parasites by flotation led to exclusion.

#### 2.2.3 | Dogs with CPV infection

Dogs were included in this group if CPV was diagnosed by fecal PCR and clinical signs were consistent with parvoviral disease. Dogs were excluded if they had received modified live CPV vaccines up to 3 weeks before to presentation. Shedding of CPV was assessed by using qualitative PCR as previously described.<sup>17</sup>

### 2.3 | Sample handling

Residual naturally passed feces remaining from previous routine fecal examination or diagnostic evaluations were examined for *netE* and *netF* by qPCR. Samples were placed in Eppendorf tubes for storage within <12 hours after collection. For dogs with AHDS and clinically healthy dogs, samples were frozen immediately at  $-80^{\circ}\text{C}$  pending analysis. Fecal samples from dogs with CPV infection were stored at  $-20^{\circ}\text{C}$ .

### 2.4 | qPCR analysis

A qPCR toxin panel was performed on each sample for the following toxin genes: *C. perfringens* alpha (*cpa*) toxin gene, *C. perfringens* enterotoxin gene (*cpe*), and both the *netE* and *netF* genes. Hydrolysis probe-based qPCR assays were designed and validated in accordance with the industry standard as previously described.<sup>18</sup> The qPCR tests were designed using a commercially available software (PrimerExpress 3.0; Thermo Fisher Scientific/Applied Biosystems, Waltham, Massachusetts; Livak et al, 1995).<sup>19</sup> Total nucleic acid from fecal samples was isolated using a previously validated protocol.<sup>20</sup> A qPCR analysis was performed on a Roche LightCycler 480, and raw data analyzed using the 2nd derivative maximum method to generate crossing points using the high sensitivity settings. The qPCR was run with 6 quality controls including (1) PCR-positive controls (synthetic DNA [Integrated DNA Technologies IDT, Coralville, Iowa], run quantitatively), (2) PCR-negative controls (RNase-free PCR-grade water, Fisher Scientific, Waltham, Massachusetts), (3) negative extraction controls (using lysis solution with spiked-in internal positive control only), (4) DNA pre-analytical quality control targeting mammalian *ssr rRNA* (18S rRNA) gene complex (quantitatively), (5) environmental contamination monitoring control (swab-based laboratory monitoring for random PCR positive signals for all toxin genes analyzed), and (6) spiked-in internal positive control

(lambda DNA). These controls assessed the functionality of the qPCR test protocols for (1) functional assessment of the toxin gene qPCR test performance, (2) the absence of contamination (both PCR product carry-over and sample cross-contamination), (3) the absence of detectable cross-contamination during the extraction process, (4) quality and integrity of the genomic DNA as a measure of sample validity (by quantitatively assessing 18S gene load), (5) the absence of aerosol-based contamination within the PCR laboratory, and (6) the absence of PCR inhibitory substances as a carryover from the sample matrix.

## 2.5 | Statistical analysis

Dogs of all 3 groups were compared for the prevalence of *C. perfringens netE* and *netF* genes. Within the AHDS group, time of hospitalization, time to recovery, and selected laboratory variables were compared between *netE*-positive and *netF*-positive and *netE*-negative and *netF*-negative dogs. Dogs were defined as clinically recovered when they showed normal demeanor, appetite, and hydration status, body temperature within the physiological range (38.0°C -39.0°C), no vomiting, and fecal consistency ≤5 according to the Purina Fecal Scoring System for Dogs (<http://www.columbusdogconnection.com/Documents/FecalScoringSystem.pdf>; accessed May 9, 2015). Data to evaluate time to recovery were available in 53 cases of AHDS.

Statistical analysis was performed using GraphPad Prism Version 6.0 (San Diego, California). Breed, sex, and the prevalence of clostridial toxin genes among dogs with AHDS, CPV infection, and healthy dogs were compared using chi-squared tests. Differences between age and the selected laboratory variables (except hematocrit and leukocytes) were analyzed using the Mann-Whitney *U* test. Comparison of time to recovery, hospitalization, hematocrit, and leukocytes was performed using an unpaired *t*-test. The level of significance was set at  $P < .05$ . A Bonferroni correction was used for multiple comparisons.

## 3 | RESULTS

Of 174 dogs included in the study, 54 had AHDS, 54 had CPV infection, and 66 dogs were clinically healthy.

### 3.1 | Signalment

The median age of the 54 dogs with AHDS was 4.0 years (range, 0.9-16.8 years). The breeds most commonly represented were mixed breed dogs ( $n = 20$ ), Yorkshire Terriers ( $n = 3$ ), and Maltese dogs ( $n = 3$ ). Thirty-one dogs were male (7 neutered) and 23 dogs were female (11 spayed). All the dogs with AHDS were routinely vaccinated, except that 3 dogs were vaccinated irregularly and 1 dog had not completed its primary immunization yet.

The median age of the 66 healthy dogs included in the control group was 4.2 years (range, 0.3-16.0 years). The most common breeds were mixed breed dogs ( $n = 32$ ), Labrador Retrievers ( $n = 7$ ), and Beagles ( $n = 3$ ). Thirty-six dogs were male (18 neutered) and 30 dogs were female (21 spayed). Sixty-four dogs were routinely vaccinated, 2 of them irregularly. In 2 cases, no information on vaccination status was available.

The median age of the 54 CPV-infected dogs was 0.17 years (range, 0.1-2.0 years). The most frequently represented breeds were mixed breed dogs ( $n = 22$ ), Labrador Retrievers ( $n = 8$ ), Maltese ( $n = 5$ ), Yorkshire Terriers ( $n = 3$ ), and Chihuahuas ( $n = 3$ ). Thirty-three dogs were male and 21 dogs were female (none neutered or spayed). Thirteen dogs were routinely vaccinated, but none of them had completed primary immunization.

Dogs with CPV infection were significantly younger compared to dogs with AHDS and clinically healthy dogs ( $P < .001$ ). No significant difference was found between dogs with AHDS and clinically healthy controls with respect to age. Breed and sex distribution was not significantly different among the 3 groups.

### 3.2 | Comparison of the prevalence of *C. perfringens netE* and *netF*, *cpa*, and *cpe* toxin genes by PCR between dogs with AHDS and controls

A significant difference ( $P < .001$ ) was found among the AHDS, CPV, and healthy control dogs with respect to the presence of *netE* and *netF* by PCR. Within the AHDS group, 26 of 54 (48.1%) dogs tested positive for *netE* and *netF*. In the CPV group, none of the dogs were positive. Within the healthy control group, 8 of 66 (12.1%) dogs were positive for *netE* and *netF* genes. The *cpe* gene was significantly less prevalent in dogs with CPV (4%) compared to dogs with AHDS (42%) and healthy dogs (32%;  $P < .001$ ). In total, 25 of 34 dogs with a positive *netE* and *netF* PCR result also were positive for the *cpe* gene. The *cpe* gene was present more frequently (42%) in dogs with AHDS than in healthy dogs (32%) with a positive *netE* and *netF* PCR, but no significant difference was found between these 2 groups ( $P = .26$ ). No difference in *cpa* detection was found among the groups ( $P > .55$ ; Table 1).

### 3.3 | Comparison of time to recovery, hospitalization time, mortality rate, and selected laboratory variables of dogs with AHDS that tested PCR positive versus negative for fecal *netE* and *netF*

No significant difference was found in time to recovery ( $P = .42$ ) and hospitalization time ( $P = .58$ ) between dogs with AHDS with and

**TABLE 1** The presence of *C. perfringens* encoding for alpha toxin gene (*cpa*), enterotoxin (*cpe*), and *netE* and *netF* toxin genes in feces of dogs with acute hemorrhagic diarrhea syndrome (AHDS), canine parvovirus infection (CPV), and healthy controls

	<i>cpa</i>	<i>cpe</i>	<i>netE</i> and <i>netF</i>
AHDS ( $n = 54$ )	32/54 (59%) <sup>a</sup>	23/54 (42%) <sup>a</sup>	26/54 (48%) <sup>a</sup>
CPV ( $n = 54$ )	36/54 (67%) <sup>b</sup>	02/54 (4%) <sup>b</sup>	00/54 (0%) <sup>b</sup>
Healthy dogs ( $n = 66$ )	43/66 (65%) <sup>c</sup>	21/66 (32%) <sup>c</sup>	08/66 (12%) <sup>c</sup>
	$P = .55^{ab}$	$P < .001^{ab}$	$P < .001^{ab}$
	$P = .57^{ac}$	$P = .26^{ac}$	$P < .001^{ac}$

A *P*-value of .05 was considered significant. Bonferroni correction  $< 0.0167$ .

<sup>a</sup>AHDS group.

<sup>b</sup>CPV group.

<sup>c</sup>Healthy group.

<sup>ab</sup>*P* value comparing group a+b.

<sup>ac</sup>*P* value comparing group a+c.

**TABLE 2** Comparison of patients with AHDS with positive (n = 26) and negative (n = 28) fecal *netE* and *netF* gene results, including time to recovery, duration of hospitalization, and laboratory parameter

Parameters	PCR positive for <i>netE</i> and <i>netF</i>		PCR negative for <i>netE</i> and <i>netF</i>		P-value
	Mean	SD	Mean	SD	
Time to recovery (d)	3.85	1.32	4.15	1.43	.42
Duration of hospitalization (d)	3.46	1.39	3.69	1.57	.57
Packed cell volume (%)	57.77	5.85	56.59	10.11	.60
White blood cells ( $\times 10^9/L$ )	11.59	3.85	12.15	4.39	.61
Banded neutrophils ( $\times 10^9/L$ )	0.889	0.698	0.971	0.768	.87
Platelets ( $\times 10^9/L$ )	324.0	114.0	355.5	100.7	.21
ALT (U/L)	59.19	39.51	64.29	35.74	.44
ALP (U/L)	59.58	55.29	61.00	62.21	.56
Bilirubin ( $\mu\text{mol/L}$ )	2.69	1.42	2.25	1.36	.21
Total protein (g/L)	68.60	9.24	60.84	9.63	.26
Albumin (g/L)	35.53	4.78	44.41	50.21	.91
Glucose (mmol/L)	5.74	1.34	5.31	1.45	.17

Abbreviations: AHDS, acute hemorrhagic diarrhea syndrome; ALP, alkaline phosphatase; ALT, alanine aminotransferase; n, number of dogs. A P-value of .05 was considered significant.

without detection of *netE* and *netF* by PCR. In addition, no significant difference in any of the following laboratory variables was present between these 2 groups of dogs with AHDS: hematocrit, numbers of white blood cells, band neutrophils, and platelets, as well as serum bilirubin, albumin, total protein and glucose concentration, and serum alanine aminotransferase and alkaline phosphatase activities (Table 2). Mortality rate was nil; 0 of 26 dogs died in the AHDS group positive and 0 of 28 dogs died in the AHDS group negative for the pore-forming toxins.

## 4 | DISCUSSION

Ours is the 1st study to evaluate the prevalence of *C. perfringens netE* and *netF* by fecal PCR in dogs with AHDS and comparing it with the prevalence in healthy dogs and dogs with CPV infection. Although a pathogenic role of *C. perfringens* in dogs with AHDS has been suspected<sup>2</sup> and epithelial necrosis might be explained by the influence of clostridial toxins, it has not been clear which toxins might be responsible for the epithelial lesions in these cases.<sup>2,7</sup> Recently, it has been shown that *netF*-producing *C. perfringens* is highly associated with fatal acute hemorrhagic enteritis in dogs and necrotizing enteritis in foals.<sup>4</sup>

A real-time PCR has been developed for both *netE* and *netF*. Recently, we showed that the *netF* gene could only be detected in *C. perfringens* type A strains isolated from the duodenum of 5 dogs with AHDS but not in clostridial strains isolated from the duodenum of 2 control dogs.<sup>21</sup> Despite better understanding of the role of *C. perfringens*, AHDS is still a diagnosis of exclusion and likely additional causes of AHDS exist. A well-recognized aspect of AHDS is its acute onset and rapid recovery of affected dogs.<sup>22,23</sup> Further work is required to investigate the dynamics of infection with *netE* and *netF* strains of *C. perfringens* and to identify additional causes of AHDS.

Clostridial species can proliferate secondarily in response to disruption of the normal gastrointestinal microbiota caused by various acute gastrointestinal disorders.<sup>8,24</sup> For this reason, we included dogs with hemorrhagic diarrhea associated with CPV infection,

representing a control group with similar epithelial necrosis as observed in dogs with AHDS. Because *C. perfringens* encoding for pore-forming toxins could not be detected in dogs with CPV infection, we conclude that *netE* and *netF*-specific toxigenic *C. perfringens* strains are not present independently of the cause of the intestinal lesions. The *cpa* qPCR showed that *C. perfringens* was found in similar prevalence and abundance in all 3 groups of dogs (Table 1). In contrast, *C. perfringens* encoding for *netE* and *netF* could not be identified in our hemorrhagic diarrhea model represented by CPV-infected dogs. These findings show that a secondary alteration of specific toxigenic *C. perfringens* is not a general sequel in all cases of acute hemorrhagic diarrhea, independent of the underlying cause. Moreover, it suggests that the association of *C. perfringens* with AHDS is the result of specific proliferation of *netE*- and *netF*-encoding strains.

Interestingly, *C. perfringens* type A strains encoding *cpa* and *cpe* can be identified in dogs with CPV infection, but *netE* and *netF* genes were detected in none of these dogs. Also, an unexpected discrepancy was found between the prevalence of *cpe* and of *netE* and *netF* genes in qPCR tests of dogs with AHDS. Slightly fewer *cpe*-positive *C. perfringens* strains (23/54) were found compared to *netE* and *netF* strains (26/54). In previous studies, *cpe*, which is present on a *tcp*-conjugative plasmid distinct from the pNetF plasmid, consistently was found in all *netF*-positive *C. perfringens* strains.<sup>4,13,25</sup>

We could not detect *C. perfringens* encoding for *netE* and *netF* in any dog with CPV infection. A methodological error is unlikely because *C. perfringens* strains encoding *cpa* could be identified in a large number of dogs with CPV infection (36/54) by PCR, and real-time PCR was performed using 6 quality controls, and the PCR method to detect *netE* and *netF* genes was equivalent to that of the AHDS and healthy control groups. The absence of *netE* and *netF*-toxigenic *C. perfringens* in CPV-infected dogs, in contrast to the 12% prevalence in the feces of healthy dogs, suggests that an unknown mechanism such as bacteriophages could be responsible for the suppression or elimination of these bacterial strains in CPV-infected dogs. Alternatively, coinfection of CPV and *netE/netF* toxigenic *C. perfringens* strains could cause an accelerated peracute clinical



course that would remove them from the patient population of the clinic, as has been suggested before.<sup>26</sup> The 12% prevalence of *netE* and *netF* strains detected by qPCR in the feces of healthy dogs is generally similar to the 10% prevalence of these strains detected by culture in the feces of dogs with nonspecific diarrheal illness<sup>4</sup> and suggests that these strains may have an association with healthy dogs. By contrast, no such strains were detected by culture in the feces of 88 young foals in Ontario subjected to one or more repeated isolation attempts.<sup>27</sup> Further work is required to understand the main source(s) of these strains for dogs and the circumstances under which they cause AHDS in dogs.

Since the discovery of *netF*-positive *C. perfringens*, many clinicians receive *netF* results as 1 variable in the so-called "diarrhea panels" but are challenged to make the right interpretation and therapeutic decisions, particularly because the genes also may be found at lower frequency in the feces of healthy dogs. A positive *netF* gene result confirms that these toxigenic *C. perfringens* strains are present and, in association with hemorrhagic diarrhea, represents a single diagnostic component supporting the diagnosis of *C. perfringens*-associated AHDS. An important finding of our study is that a positive fecal *net* gene qPCR result is not associated with a worse clinical course (Table 2). It suggests that decisions on treatment should not be based on the qualitative assessment of *netF* toxin gene results. In particular, the detection of these toxigenic clostridial strains in dogs with AHDS does not support the use of antibiotics. Two independent blinded placebo-controlled studies showed no benefit from antibiotic treatment in dogs with uncomplicated AHDS.<sup>14,28</sup>

In conclusion, our results suggest that *C. perfringens* strains encoding for *netE* and *netF* genes may play a role in dogs with AHDS. Because AHDS is a dynamic and often self-limiting acute intestinal disease, and no differences in time to recovery, duration of hospitalization, or outcome were found between *netF*-positive and *netF*-negative dogs with AHDS, a decision about antibiotic treatment should not be based solely on a positive fecal PCR result. These findings provide the basis for further work on the role of *netE/netF*-positive *C. perfringens* in AHDS in dogs, and perhaps on diarrheal illness in dogs in general, but especially on the epidemiology of AHDS and on ways it can be prevented.

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## CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

## OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

## INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Prospective collection and analysis of canine fecal samples was approved by the Ethics Committee of the Centre of Veterinary Medicine, Ludwig-Maximilian-University, Munich, Germany (approval number 7-20-06-13).

## HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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